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Seasonal dynamics of extremely halophilic microbial communities in three Argentinian salterns

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Abstract

A seasonal sampling was carried out in three Argentinian salterns: Salitral Negro (SN), Colorada Grande (CG) and Guatraché (G), aimed to analyze abiotic parameters and microbial diversity and dynamics. Microbial assemblages were correlated to environmental factors by statistical analyses. Principal-Component-Analysis of environmental data grouped SN and CG, separately from G samples, owing to its higher pH values and sulfate concentration. Differences in microbial assemblages were also found. Many archaeal sequences belonged to uncultured members of *Haloquadratum* and *Haloquadratum*-related genera, with different environmental optima. Notably, nearly half of the archaeal sequences affiliated to the recently described '*Candidatus Haloredividus*' (phylum *Nanohaloarchaeota*), not previously detected in salt-saturated environments. Most bacterial sequences belonged to *Salinibacter* representatives, while sequences affiliated to the recently described genus *Spiribacter* were also found. Seasonal analysis showed at least 40% of the microbiota from the three salterns was prevalent along the year, indicating they are well adapted to environmental fluctuations. On the other side, a minority of archaeal and bacterial sequences were found to be seasonally distributed.

Five viral morphotypes and also eukaryal predators were detected, suggesting different mechanisms for controlling prokaryotic numbers. Notably, Guatraché was the saltern which harbored the highest virus-to-cell ratios reported to date for hypersaline environments.

Introduction

Hypersaline environments are distributed all around the world (Antón *et al*. 1999; Antón *et al*. 2008; Boujelben *et al*. 2012; Demergasso *et al*. 2004; Jiang *et al*. 2007; Maturrano *et al*. 2006; Menes *et al*. 2011; Mutlu *et al*. 2008; Trigui *et al*. 2011; Zafrilla *et al*. 2010) and are influenced by very different environmental conditions, as low or high temperatures in the case of hypersaline lakes in Antarctica (Bowman *et al*. 2000) or solar salterns in the Mediterranean coasts and the Dead Sea, respectively. Within hypersaline ecosystems, talassohaline brines are those exhibiting an ionic proportion similar to that of seawater and have been classically considered as environments dominated by euryarchaeal members of the class *Halobacteria* (revised by Gupta *et al*. 2015) (being the 'square' archaeon *Haloquadratum walsbyi* the most abundant and widespread representative), which coexist with halophiles belonging to *Bacteria* (like the *Bacteroidetes* bacterium *Salinibacter ruber*) and *Eukarya* (like the algae *Dunaliella* and the brine shrimp *Artemia salina*, among others) (Antón *et al*. 2002; Antón *et al*. 2008; Bolhius *et al*. 2004; Bolhius *et al*. 2006; Boujelben *et al*. 2012; Burns *et al*. 2004; Dyall-Smith *et al*. 2011; Ochsenreiter *et al*. 2002; Oren 2008; Riddle *et al*. 2013; Ventosa *et al*. 2014; Ventosa *et al*. 2015; Wharton 2007). However, in the recent years, culture-independent studies have reported that haloarchaea from the phylum *Nanohaloarchaeota* (within the new proposed archaeal superphylum 'DPANN') as well as other *Bacteroidetes* and low GC *Actinobacteria* are also significantly abundant in these ecosystems as part of the uncultured assemblage or 'microbial dark matter' (Ghai *et al*. 2011; Gomariz *et al*. 2014; Jiang *et al*. 2007; Narasingarao *et al*. 2011; Podell *et al*. 2013; Rinke *et al*. 2013; Wang *et al*. 2011).

Together with microbial communities, halophilic viruses or 'haloviruses' (which mainly infect halophilic prokaryotes), are the other relevant biotic component in aquatic hypersaline environments, where they can reach up to 10^9 VLP (virus-like particles) per ml (Santos *et al*. 2012). Haloviruses are considered to carry out an active role in the regulation of microbial populations in close to saturation brines given that bacterivory (interpreted as "*predation on both bacteria and archaea*", as suggested by Pedrós-Alió *et al*. 2000) normally disappears above 25% salts (Guixa-Boixareu *et al*. 1996). Despite their abundance, only around 113 haloviruses have been isolated from infected cultures of hyperhalophilic microbial hosts in the last 40 years (Dyall-Smith *et al*. 2003; Roine and Oksanen 2011; Sabet 2012; Villamor *et al*. in prep.). Most of them are tailed viruses that infect haloarchaea, although other viral morphotypes (icosahedral, spindle-shaped, filamentous or pleomorphic) have also been reported from infected cultured hosts or environmental samples (Oksanen *et al*. 2012; Pietilä *et al*. 2016; Santos *et al*. 2007; Senčilo *et al*. 2012; Sime-Ngando *et al*. 2011). Given that many halophilic hosts have not been yet cultivated or they have been recently isolated, culture-independent approaches like metagenomics or single cell genomics combined with microarrays have also been applied to the study of haloviruses and virus-host interactions in hypersaline environments (Martínez-García *et al*. 2014; Santos *et al*. 2012 and references therein). Viral metagenomes (or metaviromes) from hypersaline environments have confirmed that haloviral communities are highly diverse and dynamic, reflecting their potential capacity to co-evolve together with their hosts in nature (Rodríguez-Brito *et al*. 2010).

In this work we have studied the microbial dynamics of Salitral Negro (SN), La Colorada Grande (CG) and Guatraché (G), three hypersaline salterns located in soil depressions in the Southeastern of La Pampa province, Argentina (**Fig. 1**). These salterns are currently very important working mines under commercial exploitation for the production of sodium chloride (SN and CG) and sodium sulfate (G) (website Segemar). Guatraché is fed by rain water through two influent rivers (Mining Report, 2006) while SN and CG are closed basins with ancient salt deposits originated by the evaporation of spring waters. It has also been proposed that the flow of sediments washing from the surrounding areas may help to increase the salt content of these two salterns. Besides some

technical reports focused on the geology of these environments (Mining Report 2006) and the recently reported isolation and identification of several microorganisms producing molecules with potential biotechnological application (Nercessian *et al.* 2015) there is a lack of knowledge about the microbial composition of these sites. In this study, a seasonal sampling was carried out during 2010-2011, aimed to analyze abiotic parameters (pH, temperature, total salinity and composition of most abundant ions) as well as the microbial diversity and dynamics in these three salt-saturated environments. Microbial composition was evaluated by fluorescence *in situ* hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) of the small subunit (SSU) rRNA gene PCR products amplified from environmental DNA. In addition, haloviral communities were studied by transmission electron microscopy (TEM) and pulsed-field gel electrophoresis (PFGE). Finally, data were statistically analyzed in order to give robustness to the obtained parameters. As a result, we report a deep view about the physicochemical characteristics and microbial composition and dynamics of three Argentinian brines, which demonstrate that uncultured members of *Bacteroidetes* and *Nanohaloarchaeaota* are actually ubiquitous and prevalent microbiota in most hypersaline environments, and that ionic composition is a key factor in the distribution of certain halophilic populations, what lead us to state that generalizations about microbial communities inhabiting hypersaline environments with different physicochemical traits should be carefully considered.

Materials and Methods

Sampling and physicochemical characterization

Samples were collected in autumn (03 May), winter (21 July), spring (07 October) 2010 and summer (12 January) 2011 in Salitral Negro (38º43´01´´S, 64º09´01´´W), La Colorada Grande (38º15´0´´S, 63º45´0´´W) and Guatraché (37º43´48´´S, 63º31´49´´W) salterns. For every sample, salinity, water temperature and pH values were measured *in situ*. Salinity was determined with an optical hand refractometer Atago S28-E. Subsamples were sent to the

research technical facilities at the University of Alicante (Spain) to determine their ionic compositions by High Performance Liquid Chromatography (HPLC) method using a 1260 Infinity II LC System (Agilent).

Microbial abundances

Aliquots from the different samples were fixed with formaldheyde (7% final concentration) for 16 h at 4ºC, diluted with sterile phosphate buffer solution (PBS) (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4; pH 7.4) and then filtered by 0.22 µm GTTP filters (Millipore). Hybridizations with specific probes for *Archaea* (ARCH915; Stahl and Amann 1991) and *Bacteria* (EUB338; Amann *et al*. 1990) were performed according to Antón *et al*. (1999). For total cell counts the filters were also stained with 4', 6-diamidino-2-phenylindole (DAPI). Stained cells were counted in an epifluorescence microscope (Leica, type DM4000B; Vashaw Scientifics Inc., Norcross, GA). For virus counts, aliquots from each sample were fixed with formaldehyde (4% final concentration) for 30 minutes at 4ºC, diluted with sterile milli-Q water and filtered through 0.02 µm pore size Anodisc 25 filters (Whatman). Filters were then stained with Sybr Gold (Invitrogen) according to Noble and Fuhrman (1998) and VLP counting was performed in an epifluorescence microscope (Leica, type DM4000B; Vashaw Scientifics Inc., Norcross, GA).

DNA extraction, PCR and DGGE analysis of SSU rRNA genes

For DNA extraction, 40 ml from every sample were centrifuged at 17 000 *xg* during 30 min at 4ºC in a Sorvall ST 16R centrifuge, to pellet the cells. Nucleic acids were then purified following the protocol described at Mutlu *et al*. (2008). Primers targeting conserved regions of the prokaryotic 16 rRNA genes (341f-GC: 5´-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3' and 907r: 5´-CCG TCA ATT CMT TTG AGT TT-3' for *Bacteria* and 344f-GC: 5´-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC GGG GYG CAG CAG GCG CGA-3' and 907r for *Archaea*) and eukaryotic 18S rRNA genes (Euk1A: 5´-CTG GTT GAT CCT GCC AG-3' and Euk516r-GC: 5´-CGC CCG GGG CGC

GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC CAG ACT TGC CCT CC-3') were used in the corresponding PCR reactions, performed as previously described (Amann *et al.* 1990; Muyzer *et al.* 1993). A final extension step of 30 minutes at 72ºC, (according to Janse et al. 2004) and 're-conditioning PCR' reactions (according to Thompson *et al.* 2002) were carried out to minimize PCR artifacts prior to the subsequent DGGE analysis. The 're-conditioned' PCR products were purified with the GeneJET PCR Purification Kit (Thermoscientific) according to the manufacturer's recommendations. Five hundred nanograms of each purified PCR product were then loaded in acrylamide denaturing gradient gels (0.75 mm thick). DGGE was performed in 1X Tris-acetic acid-EDTA (TAE) buffer (40 mM Tris, pH 8.0; 20 mM acetic acid; 1 mM EDTA) at 60ºC and 60 V for 16 h. The denaturing gradients were 45-65% for *Archaea* and *Bacteria*, and 30-40% for *Eukarya* (100% denaturing agents corresponds to 7 M urea and 40% deionized formamide). After running, DGGE gels were stained for 30 min with Sybr Gold, visualized under UV light and photographed with a Typhoon 9410 (Amersham Biosciences) system. Selected DNA bands were excised from the gels, resuspended in 20 µl of sterile milli-Q water and incubated at 4ºC for 16 h. DNA from each band was then re-amplified, and the resulting PCR products were loaded in new DGGE gels (in order to check that they came from a single band) and purified as described above, prior to sequencing efforts by the STAB Vida service (Portugal). Obtained sequences were screened for chimeric PCR products using the online software DECIPHER (Wright *et al.* 2012). Non-chimeric sequences were identified and taxonomically classified using the aligner tool from the Silva reference database available at http://www.arb-silva.de/aligner/. BLASTn tool at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) was used to find the closest sequences in databases. Presence/absence matrixes from DGGE profiles were used to calculate archaeal and bacterial Shannon indexes, using the Paleontological Statistics Software Package (PAST, Hammer *et al.* 2009). Cd-hit (Fu *et al.* 2012; Li and Godzik 2006) was used for the clustering of the sequences with identity percentages $\geq$ 98.7%. Obtained DGGE band sequences are deposited in the GenBank database (accession numbers KU760766 to KU760801).

Transmission Electron Microscopy (TEM) and pulsed-field gel electrophoresis (PFGE) of virus assemblages

Approximately 1 liter from each sample was centrifuged (30 000 *xg*, 30 minutes, 20°C; Avanti J-30I, Beckman with a JA rotor) in order to remove most cells. The supernatants were then concentrated until 2 ml by, sequentially: (i) tangential flow filtration through a Vivaflow system with a 30 000 MWCO (molecular weight cutoff) filter cassette and (ii) the 10 000 MWCO Amicon centrifugal filters (Millipore). Virus-enriched concentrates were finally ultracentrifuged (186 000 *xg*, for 2 h, at 20ºC in an Optima^{TM} MAX-XP Ultracentrifuge with the TLA-S5 rotor, Beckman Coulter® USA) and virus pellets resuspended in 50 µl of the same supernatant. For TEM analyses, between 0.5 and 2 microliters from each virus concentrate were stained for 45 seconds with uranyl acetate (0.5%) on Formvar-coated carbon grids (Electron Microscopy Sciences). Virus-like particles were observed in a Jeol JEM-2010 transmission electron microscope operating at 200 kV. To determine the proportion of the different viral morphotypes, between 188 and 1310 VLPs (with an average of 587 VLPs) were counted for every sample. For PFGE, approximately 5×10^8 VLPs were mixed with 1.6% low-melting-point agarose (Pronadisa), dispensed into 100-µl molds, allowed to solidify at 4°C and incubated at 50°C during 16 h in ESP (0.5M EDTA pH 9, 1% N-laurylsarcosine, 1 mg ml^{-1} proteinase K) for the disruption of viral capsids. Agarose plugs containing viral DNA were subjected to PFGE in a 1% low-electroendosmosis (LE) agarose (FMC) gel in Tris-borate-EDTA (TBE) 0.5X buffer, using a Bio-Rad (Richmond, CA) Chef DR-III system operating at 6 V cm^{-1}, with a 1- to 5-seconds pulse ramp, at 14°C for 22 h. Lambda low-range and MidRange PFG DNA size ladders (New England BioLabs) were used as molecular weight markers. The gel was visualized after staining with ethidium bromide (1 µg ml^{-1}) and photographed with a Typhoon 9410 (Amersham Biosciences) system.

Multivariate data analysis

Principal Component Analysis (PCA) of environmental parameters (salinity, ionic composition, pH and temperature) was used in order to reduce dimensionality of the data set using the SPSS software (Business Editors, 2002). Redundance Detrented Analysis (RDA), a lineal canonical multivariate

analysis method, was carried out for unveiling correlations between environmental and biological parameters (virus and cell abundance and archaeal and bacterial Shannon indexes diversity). Canonical Correspondence Analysis (CCA), a unimodal method, was used to analyze the correlation between environmental parameters and DGGE band sequences. Monte Carlo test (499 permutations) was carried out to ensure the significance of canonical axes. Multivariate analyses were performed using the CANOCO 4.5 software package and biplots were displayed by means of CANODRAW tool (ter Braak and Smilauer 2002). An ANOVA test was also performed in order to ensure the significance between environmental and biological parameters (data not shown).

Results and Discussion

Physicochemical characterization of the three salterns

Environmental parameters, including ionic composition, salinity, temperature and pH were determined for the twelve samples (**Table 1**). Salitral Negro (SN) and Colorada Grande (CG) salterns appeared as neutral basins while Guatraché (G) was slightly more alkaline. Salinity values were generally above 30%, so the ponds would act in a similar way to the crystallizers (salt-saturated ponds where sodium chloride precipitates) from coastal solar salterns. Sodium and chloride were the most abundant ions in all the samples. With regard to sulfate concentration, Guatraché presented the highest values in spring and summer samples, in agreement with the fact that this lake is exploited for the commertial production of this salt. Data from **Table 1** are in agreement with previous reports about mineral contents in the surrounding soils, which determined that sodium, chloride and sulfate were the predominant ions and that salinity remained above 29% all over the year (Mining Report 2006). The presence of all ions was not measured, which may have resulted in the differences found between the sums of cations and anions. Previous studies reported the presence of additional ions in the region (e.g., fluoride: Mining Report 2006) that were not included in these studies.

Multivariate analyses from physicochemical data (considered as explanatory variables) were performed in order to define the studied environments by means of PCA and also to correlate environmental and biological parameters (considered as dependent variables) using RDA (see below). Nine explanatory variables were reduced up to three principal components according to correlation PCA analysis (see **Table S1 in supplemental material**). Three principal components (C1-C3) explained a 75.2 % of the total variance. Calcium, sulfate and sodium defined the first component (C1), while magnesium and potassium were the most important variables in C2. Finally, temperature was the parameter exhibiting the highest significance on the third component (C3). PCA results grouped SN and CG samples together, pointing out that both salterns shared many physicochemical traits. Samples from Guatraché were clearly placed separately, indicating that they constitute an environment with different physicochemical parameters, mainly influenced by higher pH values, sulfate and sodium concentrations (see **Fig. S1 in supplemental material**).

Correlation between biological and environmental parameters

Total cell counts and relative abundances of *Archaea* and *Bacteria* are shown in **Table 1**. Most samples harbored cell numbers in the range of those previously reported for other hypersaline environments around the world (Boujelben *et al*. 2012; Demergasso *et al*. 2004; Mutlu *et al*. 2008; Trigui *et al*. 2011), being Guatraché the saltern that exhibited the highest cell values. As shown by RDA, microbial abundances were generally positively correlated to temperature while diversity indexes were positively correlated to salinity (see **Fig. 2 and Table S2 in supplemental material**), in agreement with previous trends observed in other Mediterranean and Peruvian salterns (Boujelben *et al*. 2012; Gomariz *et al*. 2014; Maturrano *et al*. 2006*)*. This statement, however, is only based on the observed general tendency after taking into account the 12 samples. It could be possible, as observed with winter samples from Colorada Grande, that particular situations at any season or saltern did not adjust to the general tendency.

FISH analyses indicated that members of *Archaea* always dominated microbial communities (**Table 1**) as it typically occurs in hypersaline environments (Antón *et al*. 1999; Jiang *et al*. 2007; Luque *et al*. 2012; Maturrano *et al*. 2006; Mutlu *et al*. 2008;), except in SN and CG summer samples, which presented a remarkable increment in bacterial cell numbers and had the lowest salinities (presumably due to the diluting effect of rains) as well as the highest calcium concentrations. Both environmental factors could be related to the increase in bacterial cell counts, as predicted by RDA analysis, which showed bacterial abundance and salinity as opposite variables and bacterial abundance and calcium concentration as positively correlated (**Fig. 2**), in accordance with previous data (Gomariz *et al*. 2014; Jiang *et al*. 2007).

The abundance of virus-like particles (VLPs) was also determined for all the samples (**Table 1**). Total VLP numbers for SN and CG were also in agreement with those previously reported for hypersaline systems, in the range of 10^8-10^9 VLP per ml (Santos *et al*. 2012). As occurred with cell counts, Guatraché samples showed the highest VLP numbers. Given that this saltern also exhibited the highest pH values, a positive dependence between viral counts and pH was observed in the RDA analysis (**Fig. 2**). As a consequence of the extremely high VLP values Guatraché samples also exhibited the highest VLP-to-cell ratios reported to date (**Table 1**), overcoming previously described values, which ranged between 42 and 100 VLPs per cell (Santos *et al*. 2012 and references therein). *In situ* viral production and decay rates analyses, together with the study of burst sizes and virus life strategies, would be needed to explain such high and stable VLP numbers.

Microbial community composition

Microbial diversity was analyzed by PCR-DGGE and sequencing of selected bands (see **Fig. S2 in supplemental material**). For archaeal and bacterial assemblages, sequences with identity percentages $\geq$ 98.7% were grouped into clusters or operationally taxonomic units (OTUs), following the criteria

established by Stackebrand and Ebers (2006), who suggested this identity threshold for the species circumscription (**Table 2**). Although many microbial ecology studies based on 16S rRNA gene sequences use a threshold of 97% identity for clustering, we opted for following a more restrictive criterion since, according to Yarza *et al.* (2014), 'exhaustive studies on determining the species thresholds indicate that a plausible species boundary would be between 98% and 99% 16S rRNA gene sequence identity at reasonable probabilities'. Moreover, since our sequences were partial and did not cover the 16S rRNA gene variable regions v1-v2, which are necessary to ascertain the species richness when the whole gene sequence is not available (Yarza *et al.* 2014), we will use the term OTU just to reflect a group of sequences belonging to the same genus, without necessarily considering them as groups of co-occurring strains within the same species.

Additionally, in order to accommodate the resulting sequences to their environmental optima, a CCA was carried out (**Fig. 3**). Sequences were arranged along environmental gradients defined by synthetic canonical axes in the resulting biplot. In the CCA, the two axes explained a 72.53% of the total variance data (see **Table S3 in supplemental material**). The first canonical ordination axis was highly correlated to pH and Na^+-Ca^{2+} concentrations while the second axis was correlated to salinity and Mg^{2+}-Cl^- concentrations. In addition, the studied samples were placed in the plot according to their physicochemical composition, as mentioned above.

As occurred with environmental parameters, and likely as a consequence of them, DGGE profiles indicated a very close similarity between SN and CG associated OTUs, which were clearly different from those from Guatraché. A prevalent prokaryotic microbiota, constituted by DGGE bands which were present along the year in each saltern was detected (52% of the analyzed bands in the case of SN, 41% for CG and 42% for G), indicating that a half of the obtained sequences were related to microorganisms well adapted to environmental fluctuations within each saltern. Remarkably, two of these bands (A08 and B02, corresponding to OTUs Hqr3 and Bdt, respectively, see below) were detected in each of the 12 analyzed samples (**Table 2**), indicating they were the

most ubiquitous and generalist prokaryotes among the three salterns. The generalist behavior of these microorganisms was also evidenced in the CCA, since these two sequences were placed in the center of the plot (**Fig. 3**).

Within the archaeal assemblage, eight OTUs (corresponding to 11 sequences) were related to members of class *Halobacteria*, within the phylum *Euryarchaeota*. Four out of these eight (Hbac1 to Hbac4) were distantly related to described members of this class, with two of them (Hbac2 and Hbac3) showing a generalist behavior and found in almost all the samples analyzed. The rest of the OTUs could be assigned to *Haloquadratum* and *Halorubrum* genera, belonging to the novel proposed family *Haloferecaceae*. OTUs Hqr1 and Hqr3 were ≥ 98% identical (**Table 2**) to uncultured members of genus *Haloquadratum*, with the type strain *Hqr. walsbyi* C23 (Burns *et al*. 2004) as the closest cultured relative, and matched with sequences also found in hypersaline environments around the globe (Baati *et al*. 2008; Dillon *et al*. 2013; Fernández *et al*. 2014; Podell *et al*. 2013). Sequences in OTU Hqr2, only detected in SN and CG, were distantly related to genus *Haloquadratum* and matched with 16S rRNA gene sequences found in Guerrero Negro salterns (Dillon *et al*. 2013) and with the 16S rRNA gene of a recently reconstructed genome retrieved from Lake Tyrrell, Australia, by metagenomics (Podell *et al*. 2013). This genome (J07HQX50), together with other 16S rRNA gene sequences found in Australian salterns (Oh *et al*. 2010), could represent a lineage separated from *Hqr. walsbyi* strains C23 and DSM 16790. Interestingly, within OTU Hqr3, sequences A07 and A08 showed different environmental optima: while A08 reflected the most generalist archaeon along the year in the three salterns, sequence A07 appeared to be more sensitive to high sulfate concentrations, as it was not detected in G samples. Unfortunately, the fact that we worked with partial sequences did not allow discerning A07 and A08 sequences as distinct strains within the same species or distinct species with the same genus. However, the fact they constitute phylogenetically cohesive, but ecologically different populations (Acinas *et al*. 2004; Cohan and Koeppel 2008), lead us to conclude that A07 and A08 sequences could represent two different ecotypes within OTU Hqr3.

Outside the *Euryarchaeota* phylum, 7 OTUs (Nah1 to Nah7, corresponding to a 42% of the bands) were related to the phylum *Nanohaloarchaeota*, which comprises uncultured and very small archaeal cells (Rinke *et al*. 2013). OTUs related to this group were mainly detected in Guatraché samples, and sequences with different environmental optima (i.e. 'ecotypes') within OTU Nah2 were also found (**Table 2 and Fig. 4**). Since Narasingarao and co-workers obtained the first two genomes of *Nanohaloarchaea* ('*Candidatus* Nanosalina sp.' and '*Candidatus* Nanosalinarum sp.') by *de novo* metagenomic assembly from Australian salterns and demonstrated that the combined abundance of these two lineages reached up to 14% of the total DAPI counts (Narasingarao *et al*. 2011), nanohaloarchaeal related sequences have been reported in several studies (Grant *et al*. 1999; Martínez-García *et al*. 2014; Oh *et al*. 2010; Pagaling *et al*. 2009; Sime-Ngando *et al*. 2011) suggesting that this group is distributed worldwide. Concurrently to the study of Narasingarao, Ghai and co-workers used the single-cell technology to analyze the microbial composition of Santa Pola salterns, in Spain. In this study an abundant nanohaloarchaeal SAG (Single Amplified Genome) was retrieved from an intermediate salinity pond (19%), and was named '*Candidatus* Haloredivivus' (Ghai *et al*. 2011). Remarkably, three out of the seven nanohaloarchaeal OTUs detected in this work (Nah5 to Nah7) were 97-99% identical to this phylotype, indicating that this group is not only present at intermediate salinities but also in close-to-saturation environments.

Among bacterial sequences, the vast majority (77%) was related to phylum *Bacteroidetes*, while the rest were affiliated to *Proteobacteria* (**Table 2**). Within the first group, seven out of eight OTUs (Sal1 to Sal7) matched with uncultured members of genus *Salinibacter,* which are considered the main bacterial players in most saturated brines (Antón *et al*. 2000; Antón *et al*. 2008; Antón *et al*. 2013). Interestingly, the closest cultured relative for almost all Sal OTUs was not *S. ruber* (the best reported and most widespread species within the genus) but *S. altiplanicus*, recently isolated from hypersaline lakes located in the Antofalla plateau, Argentina (Viver *et al*. in prep.). OTUs Sal1 to Sal4 were present mainly in SN and CG samples, in almost every season, while OTUs Sal5 to Sal7 were exclusively associated to G samples, indicating they could constitute *Salinibacter* representatives specifically adapted to high sulfate

concentrations. As in the case of archaeal OTUs Hqr3 and Nah2, sequences B07 and B11 (100% identical and grouped in OTU Sal4) showed different environmental optima, being B07 the most generalist 'ecotype', present in the three studied salterns (**Table 2 and Fig. 3**). The fact that strains with identical 16S rRNA gene sequences display different phenotypic traits and behaviors against environmental factors has indeed been investigated with *S. ruber* by metabolomics and transcriptomic approaches. In the first study (Antón *et al*. 2013), a set of 57 strains (old and newly isolated) showed a very diverse metabolic pool despite their very close phylotenetic relationship. In the second work (González-Torres *et al*. 2015) it was demonstrated that two *S. ruber* strains, co-isolated from the same saltern pond at the same time, showed distinct transcription patterns (apart from distinct accessory genes) when they were grown in co-culture, respect to the patterns displayed when growing separately in pure culture.

OTU Bdt, associated to *Bacteroidetes* but outside the *Salinibacter* group, was represented by sequence B02. This OTU was the only bacterial phylotype found along the year in each one of the three salterns analyzed (**Table 2 and Fig. 3**). Database searches indicated that this bacterium belongs to an uncultured, ubiquitous and hyperhalophilic lineage within *Bacteroidetes* phylum. Sequences related to OTU Bdt were previously found in other hypersaline environments (Benlloch *et al*. 2002; Dillon *et al*. 2013; Ghai *et al*. 2011; Jiang *et al*. 2007; Roine and Oksanen 2011; Wang *et al*. 2011; Zafrilla *et al*. 2010) and even reached up to 69% of the total bacterial clones in a salt-saturated pond from Guerrero Negro solar salterns, in Baja California (Dillon *et al*. 2013). Two years ago, the single-cell technology was applied to shed light on this unknown and ubiquitous halophilic group (Gomariz *et al*. 2014) and the analysis of the resulting SAGs revealed, among other features, that their GC content is significantly lower (47%) than that of other extremely halophilic *Bacteroidetes*, such as *Salinibacter ruber* (66%), and that members of this group could uptake DNA to face P limitation and synthetize extra ATP using light by means of a bacteriodhodopsin-like proton pump (Gomariz *et al*. 2014).

Sequence B12, exclusively found in G along the year, was affiliated with the recently described genus *Spiribacter*, within the *Gammaproteobacteria*. *Spiribacter salinus* M19-40 (León *et al*. 2014) and *Spiribacter curvatus* UAH-SP71 (León *et al*. 2015) are the only two species within the genus and were isolated from intermediate salinity ponds in Spanish solar salterns. They are chemoorganotrophic and aerobic bacteria included in the family *Ectothiorhodospiraceae*, with a 'salt out' osmoregulatory mechanism and genes coding for a type II-Xanthorhodopsin, also found in other marine *Proteobacteria*. *Spiribacter* sequences have been found worldwide in waters with salinities between 10 and 25% and the genome of the strain M19-40 was the bacterial genome which recruited the highest amount of metagenomic reads from intermediate salinity ponds from San Diego and Santa Pola salterns (López-Pérez *et al*. 2013). Interestingly, the salinity of Guatraché ranged from 33 to 37%, so this is the first report which indicates that '*Spiribacter*' representatives, as happened with archaeal OTUs Nah5, Nah6 and Nah7, can also be part of the prevalent microbiota in saturated brines.

Two bacterial OTUs (Bet1 and Bet2) were also specifically found in autumn and winter samples from G. They were affiliated with *Janthinobacterium* and *Delftia-Curvibacter* genera, within the *Betaproteobacteria*. Although betaproteobacterial sequences are normally found at low and intermediate salinities in hypersaline environments (Benlloch *et al*. 2002; Ghai *et al*. 2011; Ventosa *et al*. 2014; Ventosa *et al*. 2015) some bacterial clones related to *Janthinobacterium* sp. and one isolate from genus *Curvibacter* were retrieved, respectively, from a halite layer of athalassohaline Lake Chaka sediments (Jiang *et al*. 2007) and from an Antarctic pond underlain by hypersaline brine (Peeters *et al*. 2011).

Finally, eukaryal diversity was also analyzed by DGGE although PCR products from SN were only successfully obtained for the summer sample (**Table 2 and Fig. S2 in supplemental material**). Four bands, present in all the samples, could be successfully sequenced and one of them corresponded to the unicellular algae *Dunaliella*, the main primary producer in these environments (Oren 2002). The other three bands were associated to clones affiliated to predator

Halocafeteria (Park *et al.* 2006), a stramenopile isolated from a Korean solar saltern of 30% salinity and also found in hypersaline samples with a wide range of salinities (Park and Simpson 2015).

Description of viral assemblages

The morphologies of the viruses present in the salterns were analyzed by transmission electron microscopy after counting an average of 580 particles in every sample. TEM analyses in hypersaline environments have classically distributed the viral morphologies in four categories: icosahedral, tailed, spindle or 'lemon'-shaped and filamentous (Santos *et al.* 2012). However, unusual viral-like morphotypes (VLM) have also been detected in the Dead Sea or Lake Retba (Boujelben *et al.* 2012; Sime-Ngando *et al.* 2011). Here, the four classical VLMs were observed (**Fig. 4**). The icosahedral morphotype (VLM 1), which could also include some *Caudovirales* that lost their tails during the TEM preparations, were the most abundant VLPs in the three salterns along the year, ranging from 46 to 85% and reaching their maximum in summer samples (**Fig. 5B**). Icosahedral viruses were also the most abundant particles in the coastal Tunisian salterns of Sfax (Boujelben *et al.* 2012). Spindle-like (VLM 2) and tailed (VLM 3) viruses were generally the second and third more abundant groups (**Fig. 4 and Fig. 5B**), except in spring and summer samples of SN and CG, where filamentous particles (VLM 4) outnumbered them (see below). While tailed haloviruses have been isolated from both halobacterial and haloarchaeal hosts, the only spindle-shaped halovirus isolated to date (the halovirus His1) was obtained from an infected culture of the archaeon *Haloarcula hispanica* (Bath and Dyall-Smith 1998). Given that lemon-shaped viruses have been found to be significantly abundant in close-to-salt-saturation environments and the increase in salinity is normally accompanied by an increase in archaeal members, it has been suggested that lemon viruses would infect haloarchaea (Guixa-Boixareu *et al.* 1996). However, this trend could not be observed in the three salterns studied here since salinity values were always close to saturation in all the samples and the amount of spindle-shaped viruses were not always correlated to the

highest abundance of archaeal hosts. Filamentous viruses (VLM 4), firstly described in Spanish salterns (Santos *et al*. 2007) and proposed by Baxter and co-workers as a new haloviral morphotype in 2011 (Baxter *et al*. 2011) were among the less abundant morphologies, except in the summer samples from SN and CG, corresponding to lowest salinity values (**Fig. 4 and Fig. 5B**). The increment of this morphotype during summer samples is consistent with the increase of bacterial numbers, suggesting a positive correlation between both assemblages. Finally, a fifth morphotype (VLP 5) that could be related to spherical or membrane-enveloped viruses was also detected in these environments. Some of the spherical particles could be related to the recently described 'pleolipoviruses': haloarchaeal viruses where the virions consist of a membrane envelope surrounding the viral genome (Oksanen *et al*. 2012; Pietilä *et al*. 2016; Senčilo *et al*. 2012). However, more studies would be needed to ascertain if some of these particles corresponded to microbial vesicles, broadly described as an interspecies trafficking mechanism in microbial cultures and natural environments (Mashburn-Warren and Whiteley 2006).

In the description of viral communities, in addition to TEM, PFGE was used to study the range of genome sizes of the dominant populations (see **Fig. S3 in supplemental material**). This technique has been applied to characterize changes in the virioplankton community along a salinity gradient in Mediterranean salterns, the Dead Sea and the moderately hypersaline Mono Lake (Santos *et al*. 2012 and references therein). In these cases, authors observed viral populations with genomes of 10 to up to 500 kb, although most genomes were comprised in the range of 30 and 60 kb. Here, viral DNA genomes in the range of 33.5 to 82 kb were observed in almost every sample. Larger bands were also observed, and some of them could be related to viruses infecting eukaryal members of the community. Furthermore, although PFGE of viral assemblages has been reported to be a quick fingerprinting technique to characterize a viral community, more studies are needed to ascertain the type and topology of the nucleic acids. It is known that most isolated icosahedral and tailed haloviruses have linear dsDNA genomes. However, the genomes of the recently described 'pleolipoviruses' can be composed by a single or double stranded molecule of linear or circular DNA (Pietilä *et al*. 2016; Senčilo *et al*. 2012). Moreover, the number of genomic bands does not reflect the real diversity in a given sample,

since different viral genomes can have the same size and changes in the diversity of the viral community could be underestimated if sequencing efforts are not performed (Stewart and Azam 2000).

Virus-host dynamics

One of the models based on virus-host interactions that explain the dynamics of microbial communities, 'the kill-the-winner hypothesis' (revisited in Winter *et al*. 2010), establishes that the increase in the numbers of a given host population is followed by an increase in its corresponding virus, which acts as a predator, decreasing the abundance of the 'prey'. In the hypersaline systems studied here, this dynamic was not observed as a general trend according to global numbers. This vision, however, is very biased in complex communities since the consideration of the total number of cells as 'the host community' excludes the fine virus-host interactions that are likely occurring at the level of natural strains. Moreover, other biological interactions as bacterivory or inter- and intraspecific competition also play a role as factors controlling microbial abundances and they have not been analyzed in this work (sequences related to the predator *Halocafeteria* were detected in all the studied samples, but its abundance and active role in prokaryotic predation were not determined). In addition, high virus numbers are not always related to a high proportion of infected cells that become lysed under certain conditions. Burst sizes and virus release strategies should be considered and evaluated for a proper ecological modeling. In fact, previous works carried out in Spanish salterns revealed that, although viruses were highly active (Santos *et al*. 2011) and virus-host interactions are supposed to be very frequent due to their high numbers, the frequency of prokaryotes visibly infected by mature viruses (not considering the 'eclipse' period of the viral infection or lysogenic hosts) was low and ranged between 0.5 and 2.7% above 30% salinity (Guixa-Boixareu *et al*. 1996). The number of virions inside the cells, however, was high, reaching 35 viral particles per cell (excluding 'square' archaea) and up to 380 viral particles in the case of infected *Haloquadratum* hosts. The high numbers of VLP counted in Guatraché

samples, with prokaryotic abundances which outnumber those found in SN and CG, could be, in fact, associated to high burst sizes instead of a high portion of infected hosts (**Fig. 5**).

Regarding the dynamics of certain viral morphotypes in Argentinian salterns, halophilic *Caudovirales* (VLM 3) could start a lytic release from infected hosts in autumn, at the same time that prokaryotic populations begin decreasing, and reach a maximum in spring. Abundances of these viruses were the only that, coupled with prokaryotic abundances along the year, would follow a 'kill-the-winner' dynamics in SN and CG salterns. Spindle-like viruses could be infecting halophilic hosts better adapted to cold months and, at least in Guatraché, could be associated with haloarchaeal hosts since the proportion of lemon-shaped viruses and archaea outnumbered those found in CG and SN. If we assume that spindle-like viruses exit the cells without lysis, as it is the case of the well described halovirus His1 (Bath and Dyall-Smith 1998), their high proportion in autumn and winter would not be necessarily coupled with a decrease in archaeal numbers due to cell disruption.

With respect to the relationships between viruses and prokaryotic diversity in our samples, it was observed that high VLP-to-cell ratios, presumably as a consequence of virus release, were not affecting the diversity indexes along the year. However, since only information on microbial genera could be achieved, we were not able to unveil the effect of viral infections on the regulation of the microbial microdiversity according to the 'constant-diversity (CD) dynamics (Rodríguez-Valera 2009), which points that 'phages have a fundamental role as guarantors of the microdiversity that is required to exploit ecological resources efficiently' (Rodríguez-Valera 2009). In the CD dynamics, viruses would acquire the ability to infect new adapted host lineages preventing the replacement of inhabiting ecotypes by these new clonal populations, and following a 'Red-Queen' co-evolutionary process (revised in Liow *et al*. 2011). However, we could hypothesize that generalist microorganisms, represented by sequences A08 and B02 and associated to uncultured *Haloquadratum* and ubiquitous *Bacteroidetes*, respectively, could constitute not only the best adapted prokaryotes against environmental fluctuations, but also the best 'equipped' hosts

against viral infection, given their persistence in the systems all over the year, a trend which could also be applied to the prevalent microbiota in each one of the studied salterns.

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Table 1. Environmental and biological parameters determined in the analyzed samples.

Physicochemical parameters	Salitral Negro (SN)				Colorada Grande (CG)				Guatraché (G)			
	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter	Spring	Summer
Temperature (ºC)	21.4	2.0	18.8	22.3	13.2	11.5	27.6	38.7	17.7	8.7	28.8	45.3
pH	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.5	8.5	8.5	8.5	8.5
Salinity (%)	36.0	38.0	36.0	28.0	35.0	35.0	34.0	30.4	37.0	33.0	35.0	37.0
Na^+ (g l^{-1})	146.8	162.9	144.2	121.9	131.6	141.3	147.8	143.6	174.8	137.4	183.6	161.2
K^+ (g l^{-1})	1.50	1.00	1.80	0.30	2.00	1.60	1.10	0.54	0.80	0.90	1.00	0.96
Mg^{2+} (g l^{-1})	13.6	8.4	15.7	2.3	10.2	8.0	5.1	2.6	5.5	5.0	5.3	6.1
Ca^{2+} (g l^{-1})	0.40	0.40	0.20	1.07	0.60	0.40	0.30	0.79	0.10	0.20	0.20	0.04
Cl^- (g l^{-1})	187.4	206.6	192.8	176.1	196.1	203.5	196.3	255.4	202.6	193.3	186.8	179.7
SO_4^{2-} (g l^{-1})	23.6	19.3	34.7	8.6	20.8	16.6	10.2	8.0	22.7	21.6	53.5	76.9
Biological parameters												
Cells/ml (x 10^7)	0.7 ± 0.05	0.4 ± 0.02	1.3 ± 0.06	4.7 ± 0.13	2.1 ± 0.13	4.1 ± 0.45	0.5 ± 0.05	4.1 ± 0.18	18.4 ± 1.67	1.5 ± 0.11	1.9 ± 0.10	5.9 ± 0.37
Archaea (%)	68.6	42.8	71.7	39.5	80.3	82.9	66.7	42.6	73.9	80.0	78.9	70.8
Bacteria (%)	4.4	14.6	5.4	47.4	4.8	4.7	18.3	47.5	3.2	2.0	1.4	10.0
Non detected (%)	27.0	42.6	22.9	13.1	14.9	12.4	15.0	9.9	22.9	18.0	19.7	19.2
VLP/ml (x 10^8)	3.71 ± 0.34	3.43 ± 0.13	13.9 ± 1.47	5.43 ± 0.23	2.42 ± 0.14	2.21 ± 0.07	8.78 ± 1.29	6.11 ± 0.3	89.6 ± 0.89	34.2 ± 2.1	61.3 ± 7.39	60.6 ± 3.12
VLP to cell ratio	50.7	98.0	106.1	11.5	11.5	53.9	162.6	14.7	48.7	228.0	322.6	102.4
Archaeal diversity (H)	2.708	2.708	2.565	2.303	1.946	2.398	2.485	2.398	2.485	2.639	2.398	2.565
Bacterial diversity (H)	1.609	1.792	1.609	1.099	1.946	1.609	1.946	1.609	1.946	1.946	1.792	1.386

Table 2. Archaeal, bacterial and eukaryal DGGE sequences obtained in this study.

OTU[a]	DGGE bands (Acc. N°)	Seq. length (nt)	Ponds: season[b]	Match in SILVA database	Closest type strain (≥94.5%)[c] (Acc. N°)	Best hit in NCBI database (Acc. N°)	Isolation Source, Country (Reference)
				Archaea			
Hqr1	A01 KU760766	473	SN: AT,SP,WT CG: SP,WT G: SP,WT	98% *Euryarchaeota; Halobacteria; Haloferacales; Haloferacaceae; Haloquadratum*	98% *Hqr. walsbyi* C23 (AY676200)	99% Unc. Arch. Clone SFE1D051 (CU467201)	Sfax solar saltern, Tunisia (Baati *et al.* 2008)
Hqr2 99.80%	A02 KU760767	505	SN: AT,SM,SP,WT CG: AT,SM,SP,WT	93% *Euryarchaeota; Halobacteria; Haloferacales; Haloferacaceae; Haloquadratum*	No hits found	99% Unc. Arch. Clone P11_2-9G (KF814459)	Guerrero Negro saltern, Mexico (Dillon *et al.* 2013)
	A04 KU760768	505	SN: AT,SM,SP,WT CG: AT,SM,SP,WT	90% *Euryarchaeota; Halobacteria; Haloferacales; Haloferacaceae; Haloquadratum*	No hits found	99% Unc. Arch. Clone P11_3-3D (KF814481)	Guerrero Negro saltern, Mexico (Dillon *et al.* 2013)
Hqr3 99.17%	A07 KU760770	492	SN: AT,SM,SP,WT CG: AT,SM,SP,WT	99% *Euryarchaeota; Halobacteria; Haloferacales; Haloferacaceae; Haloquadratum*	99% *Hqr. walsbyi* C23	99% Unc. Arch. Clone J07HQW1_J07B_scf56329_01 (KF673184)	Hypersaline Lake Tyrrell, Australia (Podell *et al.* 2013)
	A08 KU760771	480	SN: AT,SM,SP,WT CG: AT,SM,SP,WT G: AT,SM,SP,WT	99% *Euryarchaeota; Halobacteria; Haloferacales; Haloferacaceae; Haloquadratum*		99% Unc. Arch. Clone IC21-C1439 (KJ588887)	Isla Cristina saltern, Spain (Fernández *et al.* 2014)
Hrr	A19 KU760781	500	SN: AT,SP,WT CG: SP G: AT,SM,SP,WT	99% *Euryarchaeota; Halobacteria; Haloferacales; Haloferacaceae; Halorubrum*	99% *Hrr. chaoviator*	99% *Hrr* sp. AJ126 (HE802596)	Andean Lakes, Argentina (Maldonado and Farias, unp.)
Hbac1	A13 KU760775	540	SN: AT,SM,SP,WT CG: AT,SM,SP,WT G: SP,WT	92% *Euryarchaeota; Halobacteria; Halobacteriales; Halobacteriaceae*	No hits found	99% Unc. Arch. DGGE band 4A (HQ455545)	Bras del Port saltern, Spain (Santos *et al.* 2011)
Hbac2	A17 KU760779	496	SN: AT,SM,SP,WT CG: SM,SP,WT G: AT,SM,SP,WT	97% *Euryarchaeota; Halobacteria; Halobacteriales; Halobacteriaceae*	97% *Halonotius pteroides* (AY498641)	99% Unc. Arch. Clone P12_9C (KF234309)	Guerrero Negro saltern, Mexico (Dillon *et al.* 2013)
Hbac3	A18 KU760780	500	SN: AT,SM,SP,WT CG: AT,SM,SP,WT G: AT,SM,SP,WT	91% *Euryarchaeota; Halobacteria; Halobacteriales; Halobacteriaceae*	No hits found	99% Unc. Arch. DGGE band 4A	Bras del Port saltern, Spain (Santos *et al.* 2011)
Hbac4 98.98%	A21 KU760783	490	SN: SM,WT CG: SM G: AT	91% *Euryarchaeota; Halobacteria; Halobacteriales; Halobacteriaceae*	No hits found	98% Unc. *Halobacterium* clone SFH1C111 (FN391271)	Sfax solar saltern sediment, Tunisia (Baati *et al.* 2010)

	A22 KU760784	497	SN: AT,SM,SP,WT CG: SM,SP,WT G: AT,SM,SP				
Nah1	A05 KU760769	511	SN: AT,SM,SP,WT CG: SM,SP	89% *Nanohaloarchaeota*	No hits found	98% Unc. Arch. Clone XH57 (FM210874)	Hypersaline Lake, China (Pagading *et al.* 2009)
Nah2 100%	A09 KU760772	542	SN: AT,SM,SP,WT CG: SM,SP,WT G: SM,WT	88% *Nanohaloarchaeota*	No hits found	98% Unc. Arch. Clone CV11 (HQ197755)	Hypersaline Lake Tyrrell, Australia (*Narasingarao et al.* 2011)
	A20 KU760782	437	G: AT,SM,SP,WT				
Nah3	A11 KU760773	494	SN: AT CG: AT G: SM,SP	86% *Nanohaloarchaeota*	No hits found	96% Unc. Arch.- Single Amplified Genome AB578-J17 (KF771598)	Bras del Port salterns, Spain (Martínez-García *et al.* 2014)
Nah4	A12 KU760774	540	SN: AT,SP G: AT,SM,WT	88% *Nanohaloarchaeota*	No hits found	97% Unc. Arch. Clone XH57	Hypersaline Lake, China (Pagading *et al.* 2009)
Nah5	A14 KU760776	415	SN: WT G: AT,SM,WT	88% *Nanohaloarchaeota*	No hits found	98% Unc. Arch. Clone 4097 (KJ546112)	Bras del Port salterns, Spain (Ghai *et al.* 2011)
Nah6	A15 KU760777	486	G: AT,SM,WT	88% *Nanohaloarchaeota*	No hits found	97% Unc. Arch. Clone 4097	Bras del Port salterns, Spain (Ghai *et al.* 2011)
Nah7	A16 KU760778	495	G: AT,SM,SP,WT	87% *Nanohaloarchaeota;*	No hits found	99% Unc. Arch. Clone 4097	Bras del Port salterns, Spain (Ghai *et al.* 2011)
Bacteria							
Sal1	B01 KU760785	523	SN: AT,SP,WT GC: AT,SP,WT	96% *Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Rhodothermaceae; Salinibacter*	96% *Salinibacter ruber* DSM 13855 (CP000159)	99% Unc. Bact. Clone GB47 (HQ425196)	Aran-Bidgol Salt Lake, Iran (Makhdoumi-Kakhki *et al.* 2012)
Sal2 99.62%	B05 KU760789	526	SN: AT,SM,SP,WT GC: AT,SM,SP,WT	95% *Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Rhodothermaceae; Salinibacter*	95% *S. altiplanicus* (Viver et al., in prep.)	99% Unc. Bact. Clone SFC1D061 (AM981340)	Salt crystal from Tunisian solar saltern (Baati *et al.* 2010)

Sal3	B06 KU760790	521	CG: AT,SM,SP	94% *Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Rhodothermaceae; Salinibacter*	98% *S. altiplanicus*	100% Unc. Bact. Clone CBB310825771 (JX885102)	Hypersaline Lake Tyrrell, Australia (Podell *et al*. 2013)
Sal4 100%	B07 KU760791	525	SN: WT CG: AT,SM,SP,WT G: SP,WT	97% *Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Rhodothermaceae; Salinibacter*	98% *S. altiplanicus*	99% Unc. Bact. Clone 188ZA09 (FN393431)	Sfax solar saltern sediment, Tunisia (Baati *et al*. 2010)
	B11 KU760795	526	SN: AT,SM,SP,WT GC: AT,SM,SP,WT				
Sal5	B08 KU760792	520	G: AT,SP,WT	97% *Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Rhodothermaceae; Salinibacter*	96% *S. altiplanicus*	98% Unc. Bact. Clone 188ZG12 (FN393437)	Sfax solar saltern sediment, Tunisia (Baati *et al*. 2010)
Sal6	B09 KU760793	520	G: AT,SM,SP,WT	97% *Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Rhodothermaceae; Salinibacter*	97% *S. altiplanicus*	99% Unc. Bact. Clone LL68B (EF106056)	Evaporitic crust Lindsey Lake, New Mexico (Sahl *et al*. 2008)
Sal7	B10 KU760794	519	G: AT,SM,SP,WT	96% *Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Rhodothermaceae; Salinibacter*	97% *S. altiplanicus*	98% Unc. Bact. Clone CBB310788661 (JX884638)	Hypersaline Lake Tyrrell, Australia (Podell *et al*. 2013)
Bdt	B02 KU760786	524	SN: AT,SM,SP,WT CG: AT,SM,SP,WT G: AT,SM,SP,WT	83% *Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae*	No hits found	99% Unc. Bact. Clone P12_8F (KF234369)	Guerrero Negro salterns, Mexico (Dillon *et al*. 2013)
Bet1	B03 KU760787	532	G: AT,WT	100% *Proteobacteria; Betaproteobacteria; Burkholderiales; Oxalobacteraceae*	100% *Janthinobacterium lividum* (Y08846)	100% *Janthinobacterium* sp. LF3 (KT424976)	Glacier melt water, China (Li unp.)
Bet2	B04 KU760788	532	G: AT	95% *Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Delftia*	95% *Curvibacter gracilis* (AB109889)	95% Bacterium SRMC-19-8 (DQ104942)	Freshwater, USA (Stepanauskas *et al*. 2006)
Spir	B12 KU760796	532	G: AT,SM,SP,WT	97% *Proteobacteria; Gammaproteobacteria; Chromatiales; Ectothiorhodospiraceae; Spiribacter*	96% *Spiribacter salinus* (CP005963)	99% Unc. Bact. Clone P_B9 (KF234369)	Black Sea coast, Bulgaria (Tomova *et al*. unp.)

				Eukarya				
-	E01 KU760798	481	SN: SM CG: AT,SM,SP,WT G: AT,SM,SP,WT	99% *Chlorophyta; Chlorophyceae*	-	99% Unc. *Dunaliella* Clone LT37_A1 (KC486721)	Lake Tyrrell, Australia (Heidelberg *et al.* 2013)
-	E02 KU760799	490	SN: SM CG: AT,SM,SP,WT G: AT,SM,SP,WT	83% *Stramenopiles; Bicosoecida*	-	99% *Halocafeteria* sp. WVII 10/2 clone 320 (KT210066)	Hypersaline Hutt Lagoon, Australia (Park and Simpson 2015)
-	E03 KU760800	478	SN: SM CG: AT,SM,SP,WT G: AT,SM,SP,WT	83% *Stramenopiles; Bicosoecida*	-	99% *Halocafeteria* sp. H6 clone 11/12 (KT210057)	Hypersaline Hutt Lagoon, Australia (Park and Simpson 2015)
-	E04 KU760801	455	SN: SM CG: AT,SM,SP,WT G: AT,SM,SP,WT	83% *Stramenopiles; Bicosoecida*	-	100% *Halocafeteria* sp. WVII 10/2 clone 320 (KT210066)	Hypersaline Hutt Lagoon, Australia (Park and Simpson 2015)

a. DGGE band sequences within a single OTU have identity percentages $\geq$ 98.7%

b. AT: autumn; SM: summer; SP: spring; WT: winter

c. Only type strains with identity percentages $\geq$94.5% (genus threshold according to Yarza *et al.* 2014) are shown
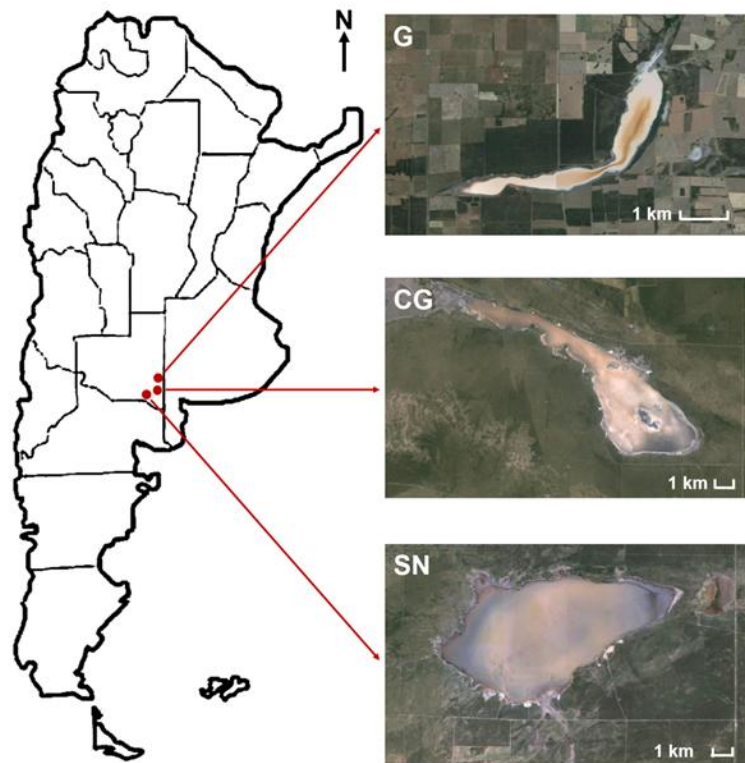
Figure 1. Location of the three salterns studied. *SN*: Salitral Negro (38º43´01´´S, 64º09´01´´W, 20 km^2), *CG*: La Colorada Grande (38º15´0´´S, 63º45´0´´ W, 56 km^2) and *G*: Guatraché (37º43´48´´S, 63º31´49´´W, 85 km^2). The distance between SN and G (the more distant places) is up to 130 km. Map data 2016 Digital Globe. Cnes/Spot Image. 2016 Google.
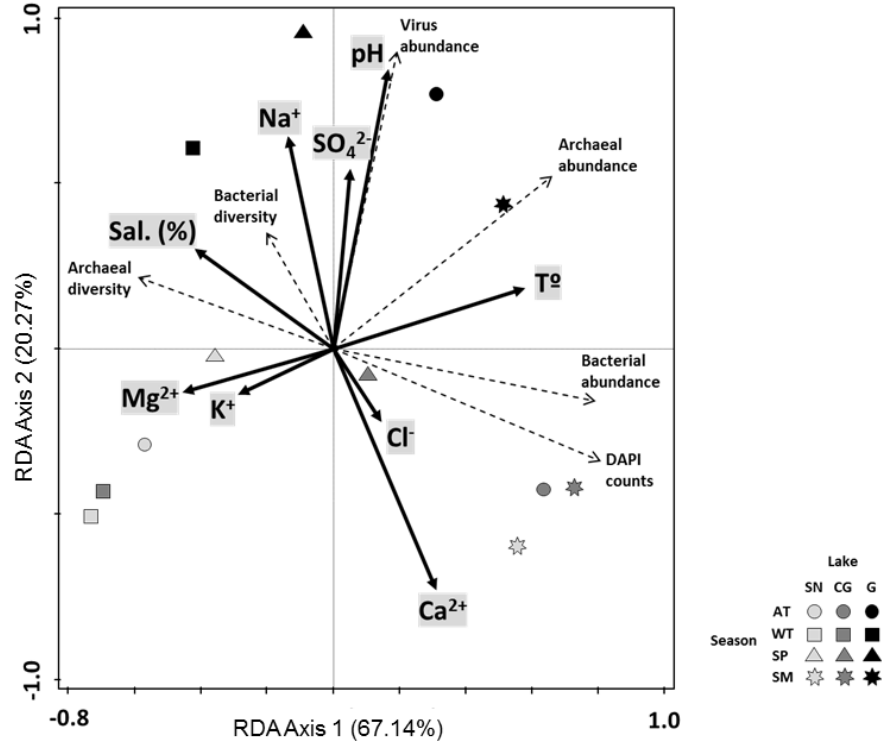
Figure 2

Figure 2. RDA biplot of environmental (black arrows) and biological (broken arrows) parameters. DAPI counts, archaeal, bacterial and virus abundances are represented by the logarithm of their numbers per milliliter. Diversity of *Archaea* and *Bacteria* are based on calculated Shannon diversity indexes. The two synthetic canonical axes (RDA Axis 1 and RDA Axis 2) explained a 87.41% of data variance (see Table S2 in supplemental material). The samples analyzed in this study (see legend) are situated in the biplot according to their relationship with environmental parameters.
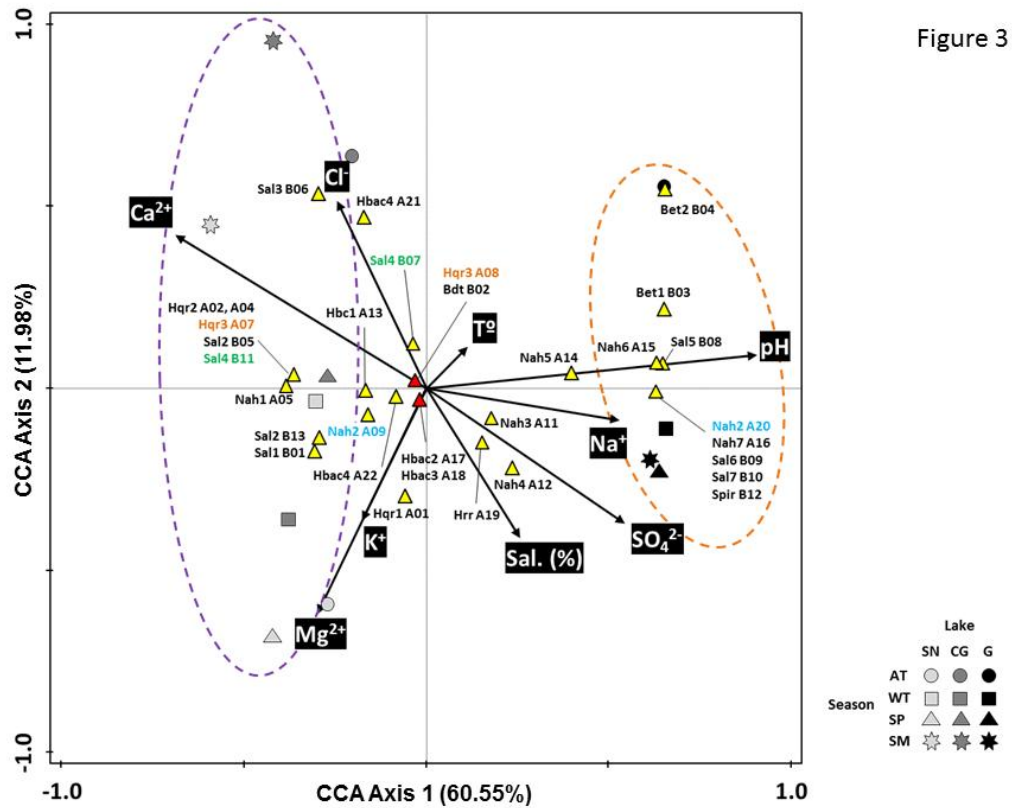
Figure 3. CCA biplot of OTUs (red/yellow triangles) and environmental parameters (black arrows). The two synthetic canonical axes (CCA Axis 1 and CCA Axis 2) explained a 72.53% of data variance (see Table S3 in supplemental material). OTUs are named as in Table 2 (name of the OTU and associated sequence/sequences). Prokaryotic biota specific for each environment is indicated by orange (Guatraché) and lilac (Salitral Negro / Colorada Grande) ovals. Red triangles in the center of the biplot reflect the most generalist microorganisms (present in the twelve analized samples and not determined by a specific

environmental factor). Colored OTUs Hqr3, Sal4 and Nah2 are formed by sequences with different environmental optima. The samples analyzed in this study (see legend) are situated in the biplot according to their relationship with environmental parameters.
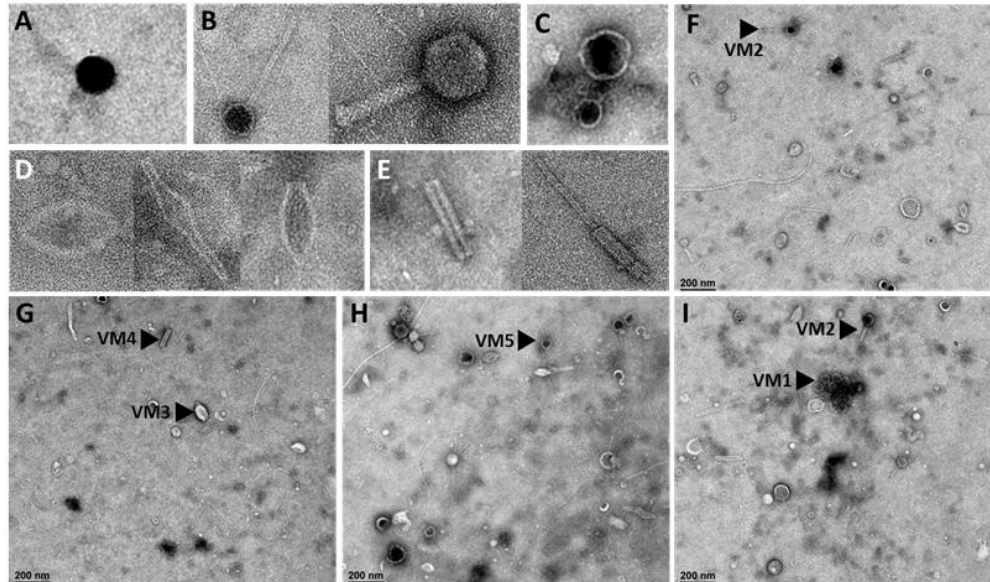
Figure 4

Figure 4. Transmission electron micrographs showing the viral morphotypes detected in this study. A: icosahedral morphotype (VM1). B: tailed morphotype (VM2). C: spherical morphotype (VM5). D: spindle-shaped morphotype (VM3). E: filamentous morphotype (VM4). F-I: virus-like particles (VLPs) in Colorada Grande winter samples (examples of viral morphotypes are indicated by arrows; scale bar: 200 nm)
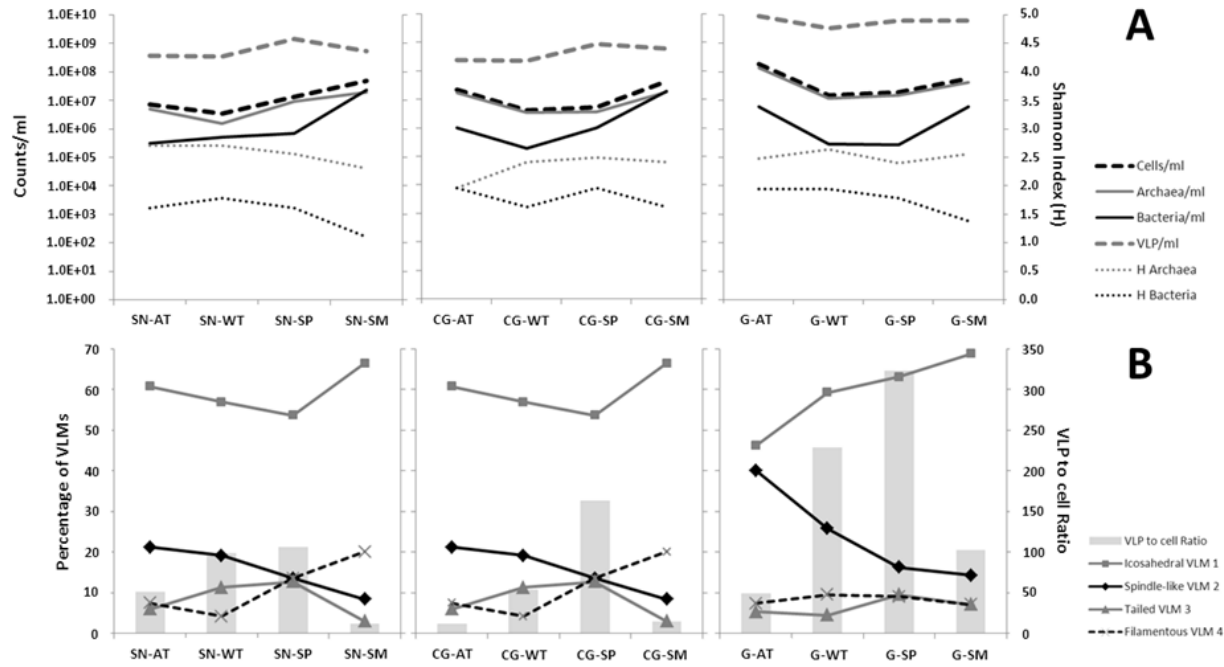
Figure 5. Integration data. A. Left axis: DAPI (total cells), FISH (*Archaea* and *Bacteria*) and Sybr-Gold (VLPs) counts per ml; right axis: Shannon diversity indexes (H) for archaeal and bacterial assemblages. B. Left axis: percentages of virus morphotypes VLM1 to VLM4; right axis: virus-like-particles to cell ratios.